

UCSF

UC San Francisco Previously Published Works

Title

Pseudomonas aeruginosa exploits a PIP3-dependent pathway to transform apical into basolateral membrane.

Permalink

<https://escholarship.org/uc/item/0dk9c33m>

Journal

The Journal of cell biology, 177(1)

ISSN

0021-9525

Authors

Kierbel, Arlinet
Gassama-Diagne, Ama
Rocha, Claudia
et al.

Publication Date

2007-04-01

DOI

10.1083/jcb.200605142

Peer reviewed

Pseudomonas aeruginosa exploits a PIP3-dependent pathway to transform apical into basolateral membrane

Arlinet Kierbel,¹ Ama Gassama-Diagne,^{3,4,6} Claudia Rocha,⁷ Lilliana Radoshevich,⁵ Joan Olson,⁷ Keith Mostov,^{3,4} and Joanne Engel^{1,2}

¹Department of Medicine, ²Department of Microbiology and Immunology, ³Department of Anatomy, ⁴Department of Biochemistry and Biophysics, and ⁵Department of Biomedical Sciences Graduate Program, University of California, San Francisco, San Francisco, CA 94143

⁶Institut Federatif de Recherche Claude de Preval, Institut Federatif de Recherche 30, Institut National de la Santé et de la Recherche Médicale Unite 563, Département Lipoprotéines et Médiateurs Lipidiques, Hôpital Purpan, 31059 Toulouse, Cedex 3, France

⁷Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425

P*seudomonas aeruginosa*, an important human pathogen, preferentially binds and enters injured cells from the basolateral (BL) surface. We previously demonstrated that activation of phosphatidylinositol 3-kinase (PI3K) and Akt are necessary and sufficient for *P. aeruginosa* entry from the apical (AP) surface and that AP addition of phosphatidylinositol 3,4,5-trisphosphate (PIP3) is sufficient to convert AP into BL membrane (Kierbel, A., A. Gassama-Diagne, K. Mostov, and J.N. Engel. 2005. *Mol. Biol. Cell.* 16:2577–2585; Gassama-Diagne, A., W. Yu, M. ter Beest, F. Martin-Belmonte, A. Kierbel, J. Engel, and K. Mostov. 2006. *Nat. Cell Biol.* 8:963–970).

We now show that *P. aeruginosa* subverts this pathway to gain entry from the AP surface. In polarized monolayers, *P. aeruginosa* binds near cell–cell junctions without compromising them where it activates and recruits PI3K to the AP surface. Membrane protrusions enriched for PIP3 and actin accumulate at the AP surface at the site of bacterial binding. These protrusions lack AP membrane markers and are comprised of BL membrane constituents, which are trafficked there by transcytosis. The end result is that this bacterium transforms AP into BL membrane, creating a local microenvironment that facilitates its colonization and entry into the mucosal barrier.

Introduction

Many internal organs are lined by a monolayer of polarized epithelia with separate apical (AP) and basolateral (BL) surfaces that are defined by distinct protein and lipid compositions and are separated by tight junctions (Gibson and Perrimon, 2003). The AP surface serves as a barrier to the outside world and is specialized for the exchange of materials with the lumen. The BL surface is adapted for interaction with other cells and for exchange with the bloodstream. Among its many roles, this epithelial barrier is one of the most fundamental components of the innate immune system, protecting organisms from the vast environmental microbiota; indeed, >90% of infectious agents

enter through mucosal epithelia. Although an effective defense mechanism against most microbes, pathogenic bacteria have evolved or acquired strategies to circumvent the mucosal barrier (Kazmierczak et al., 2001). For example, some professional pathogens such as *Salmonella typhimurium* and *Shigella flexneri* inject into the host cell toxins that subvert host signal transduction pathways and manipulate the host cell cytoskeleton in ways that allow entry through the AP surface of the mucosal barrier (Cossart and Sansonetti, 2004).

In contrast, for opportunistic pathogens, of which *Pseudomonas aeruginosa* is a prime example, the mucosal barrier represents a formidable challenge to bacteria-mediated damage or entry. However, in the setting of injured or poorly polarized epithelium, *P. aeruginosa* can initiate colonization and unleash its arsenal of potent virulence factors (Engel, 2003). Indeed, this gram-negative pathogen is a leading cause of nosocomial infections in hospitalized patients and accounts for its predilection to cause ventilator-associated pneumonia, skin infections in burn patients or at the site of surgical incisions, and catheter-related

Correspondence to Joanne Engel: jengel@medicine.ucsf.edu

J. Olson's present address is Dept. of Microbiology, Immunology, and Cell Biology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506.

Abbreviations used in this paper: AP, apical; BL, basolateral; DN, dominant negative; LY, LY294002; PH, pleckstrin homology; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate.

The online version of this article contains supplemental material.

infections, among others (Mandell et al., 2000). *P. aeruginosa* is also a cause of chronic lung infections and ultimately death in patients with cystic fibrosis (Mandell et al., 2000).

Although usually considered an extracellular pathogen, ~50% of all isolates can be measurably internalized into non-phagocytic cells in vivo as well as in vitro (Engel, 2003). A seemingly simple but very important question is from what surface *P. aeruginosa* optimally enters the host cells. In tissue culture models, *P. aeruginosa* is observed to preferentially bind to and enter the cells at the edge and BL surfaces at the site of mechanical wounding, corresponding to injured and poorly polarized cells (Geiser et al., 2001). Consistent with this, we have found that several strains of *P. aeruginosa* enter more efficiently into incompletely polarized cells (Kazmierczak et al., 2004; unpublished data).

Phosphatidylinositol 3,4,5-trisphosphate (PIP3) has recently emerged as both a key determinant of epithelial polarity and of pathogen interaction with host cells (Vanhaesebroeck and Alessi, 2000; Wymann et al., 2003; Pizarro-Cerda and Cossart, 2004). In MDCK cells, a well-studied model of polarized epithelium, PIP3 is stably localized at the BL membrane and is excluded from the AP plasma membrane (Watton and Downward, 1999; Gassama-Diagne et al., 2006). The mechanism by which a gradient of this freely diffusible lipid is maintained has not been fully elucidated, but it most likely involves localization of the lipid phosphatase PTEN (phosphatase and tensin homologue)

to the tight junction (von Stein et al., 2005). Phosphatidylinositol 3-kinase (PI3K) induces scattering and tubulogenesis in epithelial cells through a novel pathway (Yu et al., 2003). We have recently shown that PIP3 plays a key role in determining the composition and identity of the BL surface (Gassama-Diagne et al., 2006). Insertion of exogenous PIP3 into the AP surface results in the rapid transformation of regions of the AP surface into a membrane with the composition of the BL surface by redirecting BL transcytosis. Conversely, reduction in the synthesis of PIP3 by the inhibition of PI3K causes a decrease in BL surface area. Together, these results suggest that PIP3 is necessary and sufficient for the specification of BL membrane. Interestingly, PIP3 is also involved in morphogenesis of the AP surface of photoreceptor cells in *Drosophila melanogaster* (Pinal et al., 2006).

In a previous study, we have discovered that the PI3K pathway is necessary and sufficient for the internalization of *P. aeruginosa* into epithelial cells (Kierbel et al., 2005). In the present study, we demonstrate that this pathogen subverts PI3K to alter membrane polarity. We show that in response to *P. aeruginosa* binding to the AP surface, many BL proteins as well as PI3K and actin are rapidly redistributed to the regions of bacterial binding at the AP surface without disrupting the integrity of the tight junctions. Concurrently, proteins that are normally resident at the AP surface are removed from the regions of bacterial attachment. We demonstrate that BL constituents of the

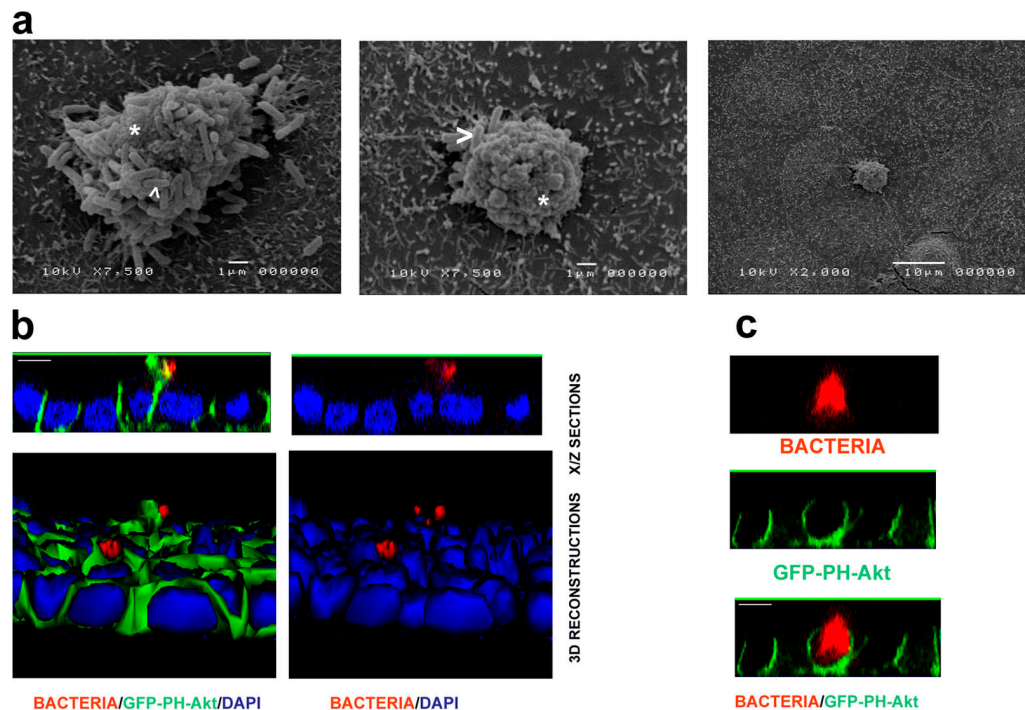


Figure 1. Attachment of *P. aeruginosa* to the AP membrane of MDCK cells triggers the formation of membrane protrusions. (a) Scanning electron microscopy of MDCK cells infected with *P. aeruginosa* for 30 min. (left and middle) Top-down views of membrane protrusions. The host membrane is indicated by asterisks, whereas bacteria are indicated by the arrowheads. (right) Lower magnification of the picture in the middle panel showing that bacteria were typically observed to adhere to the cell-cell junctions. (b and c) Confocal micrographs of MDCK cells expressing GFP-PH-Akt (green; a marker for PIP3) infected with PKH26 red-labeled *P. aeruginosa*. (b) XZ sections and 3D reconstruction of samples fixed 30 min after the addition of bacteria. The host cell nuclei are stained with DAPI (blue). PIP3-containing protrusions are observed at the site of bacterial binding to the AP surface. The MDCK cells form a single monolayer of cells. (c) XZ sections of samples fixed 2 h after the addition of bacteria. The bacteria were allowed to adhere for 1 h, nonadherent bacteria were removed by washing, and extracellular bacteria were killed by the addition of amikacin for 1 h. The bacteria are fully internalized; no AP protrusions or AP PIP3 are observed. Bars, 10 μ m.

protrusions originate from the BL membrane and are redirected to the AP membrane by a dynamin- and PI3K-dependent transport mechanism likely involving transcytosis. By transforming an AP surface into one with BL characteristics, the bacteria create a local microenvironment that facilitates colonization and entry into the mucosal barrier.

Results and discussion

P. aeruginosa induces the formation of protrusions on the AP surface near cell-cell junctions

In polarized MDCK cells, the pleckstrin homology (PH) domain of Akt fused to GFP (PH-Akt-GFP), a protein probe for PIP3, localizes exclusively at the BL surface in an LY294002 (LY)-dependent manner, whereas a point mutant unable to bind 3' phosphoinositides is cytoplasmic (Watton and Downward, 1999; unpublished data). We have previously shown that upon AP *P. aeruginosa* infection, PH-Akt-GFP relocated to the site of AP bacterial binding (Kierbel et al., 2005). This process as well as subsequent bacterial entry was blocked by the PI3K inhibitor LY, suggesting a key role for PI3K in these events (Kierbel et al., 2005). These findings suggested that the bacteria induce the relocation of lipids normally found only at the BL surface (Watton and Downward, 1999; Yu et al., 2003).

As the PH domain of Akt can also bind to phosphatidylinositol 3,4-bisphosphate, we confirmed that PIP3 was recruited to the site of AP bacterial binding using a more specific probe, the PH domain of Grp1 fused to GFP (Klarlund et al., 1997, 1998). Polarized confluent MDCK cells grown on transwell filters were transiently transfected with PH-Grp1-GFP and infected with *P. aeruginosa* (MOI of 50) for 30 min and fixed. Similar to our results with PH-Akt-GFP, this PIP3 probe showed a BL distribution and localized to the bacterial binding site (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200605142/DC1>). We further investigated the spatial relationship of the bacteria to the cell surface by scanning electron microscopy. Extensive membrane protrusions from the AP surface were observed at the site of the attachment of bacterial aggregates (Fig. 1 a). Most of the bacterial binding occurred at or near cell-cell junctions (Fig. 1 a, right). As *P. aeruginosa* preferentially binds to BL surfaces when they are accessible, we considered the possibility that the bacteria were binding to BL surfaces of areas of multilayered cells. MDCK cells stably transfected with GFP-PH-Akt were cocultivated with PKH26 red-labeled *P. aeruginosa* for 30 min. In general, PIP3-containing protrusions were not associated with multilayered cells (Fig. 1 b). By 2 h, a time at which bacterial internalization is complete, protrusions were no longer visible (Fig. 1 c).

The bacteria-induced protrusions contain PI3K and actin

We investigated whether PI3K, the enzyme that generates PIP3, is recruited to the AP protrusions. MDCK cell monolayers were infected with bacteria for 30 min, fixed, stained with an antibody to PI3K, and examined by confocal microscopy. In uninfected cells, cytoplasmic PI3K was preferentially found in the

BL regions of the cells (unpublished data). Within 30 min after the addition of bacteria, PI3K accumulated at the AP surface underneath the site of bacterial attachment (Fig. 2 a).

Membrane protrusion and generation of PIP3 are events often associated with actin rearrangement (Pollard and Borisy, 2003), and *P. aeruginosa* entry is inhibited by cytochalasin D (Evans et al., 1998). Phalloidin staining revealed filamentous actin in the bacteria-induced protrusions (Fig. 2 b). Protrusion formation was completely blocked by latrunculin B, indicating that their formation requires filamentous actin (unpublished data).

P. aeruginosa transforms AP membrane into BL membrane during attachment to the AP surface of polarized MDCK cells

We examined the membrane constituents of the bacteria-induced protrusions using antibodies to AP and BL proteins. Surprisingly, gp135/podocalyxin, a component of AP membranes, was absent from the PIP3-rich protrusions (Fig. 3 a). Confocal XZ sections revealed the acquisition of the BL markers p58, β -catenin (Fig. 3, b and c), and β 1-integrin (not depicted). These remarkable changes in membrane composition are evident in the 3D reconstructions, which clearly show the bacteria aggregates associated with protrusions of the AP membrane that contain BL proteins and exclude AP proteins. Interestingly, the PIP3 probe GFP-PH-Akt and staining of the BL markers did not completely overlap, which may reflect the relative rapid degradation of PIP3 at the AP membrane by lipid phosphatases (von Stein et al., 2005). Together, these experiments reveal that binding of *P. aeruginosa* to the AP surface of the epithelial barrier is able to radically alter the composition of the membrane, transforming it from an AP surface to one with BL constituents.

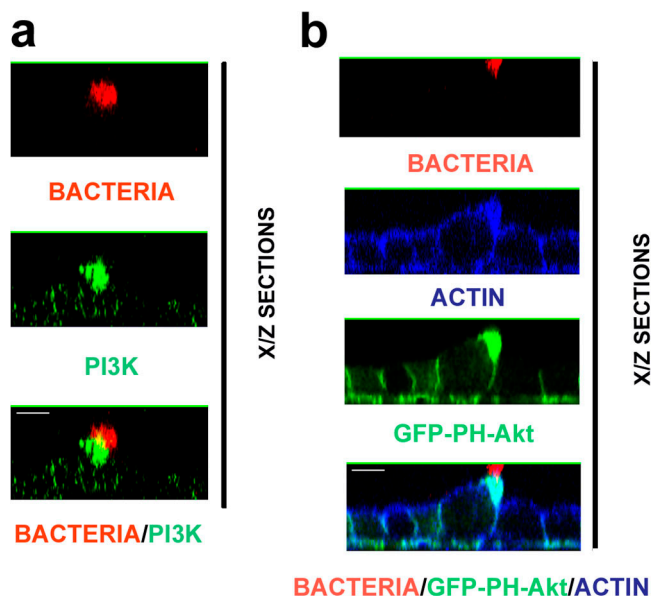
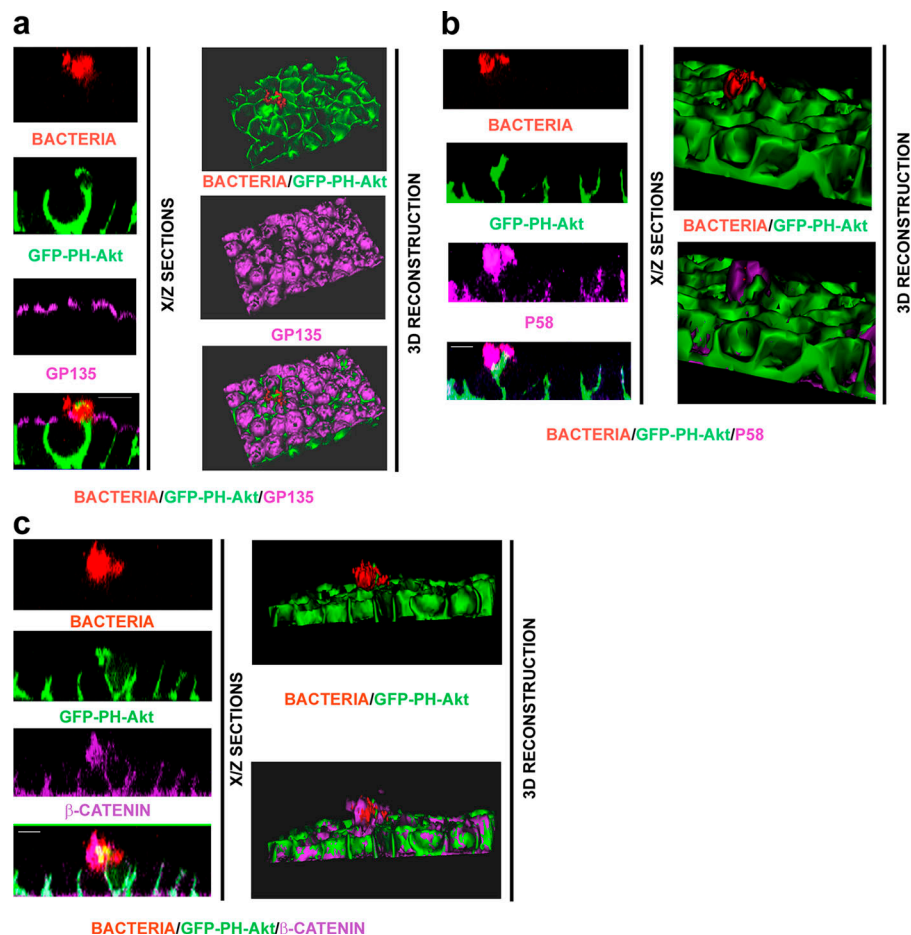


Figure 2. PI3K and filamentous actin are recruited to the protrusions at the site of bacterial attachment. Confocal micrographs of wild-type MDCK (a) or MDCK cells expressing GFP-PH-Akt infected with PKH26 red-labeled *P. aeruginosa* for 30 min (b). XZ sections revealed the recruitment of PI3K (green; a) and filamentous actin (blue; b) to the bacteria-induced protrusions at the site of AP binding. Bars, 10 μ m.

Figure 3. *P. aeruginosa*-triggered AP protrusions have BL membrane characteristics. XZ and 3D reconstructions of confocal micrographs of MDCK cells expressing GFP-PH-Akt infected with PKH26 red-labeled *P. aeruginosa* for 30 min showing both vertical (XZ) sections as well as 3D reconstructions of a portion of the monolayer. (a) PIP3 (GFP-PH-Akt; green) is recruited to the AP bacteria-induced protrusion, whereas the AP protein gp135 (purple) is absent. (b and c) The BL marker proteins p58 (purple; b) and β -catenin (purple; c) are recruited to the AP bacteria-induced protrusions. The 3D reconstructions show that BL components appear to surround the bacterial aggregate. Bars, 10 μ m.



This transformation is accompanied by the recruitment of PI3K, the generation of PIP3 at the AP surface, and the recruitment of actin into membrane protrusions. AP proteins are lost from protrusions, which instead become enriched in BL proteins. These observations rule out the possibility that BL proteins are simply intermixed with AP proteins in these protrusions.

BL membrane constituents are recruited to the AP protrusion by BL to AP transcytosis

Proteins normally destined for the BL surface could be redirected to the AP membrane from intracellular stores or from the BL membrane. To distinguish between these two possibilities, we used a pulse-chase experiment to investigate whether proteins from the BL surface were present in the AP protrusion. BL surface molecules were selectively biotinylated at 4°C by the addition of sulfo-*N*-hydroxysulfosuccinimide-biotin to the BL medium. This compound reacts with free amino groups on the surface but does not cross the tight junctions (Sadoul et al., 1995). *P. aeruginosa* was added to the AP surface, and biotinylated BL molecules were detected by fluorescent streptavidin. As seen in Fig. 4 a (left), biotinylated BL proteins were detectable in the AP protrusions. In contrast, AP staining was not detected in adjacent or uninfected cells (unpublished data).

This experiment suggests that BL constituents of the AP protrusions originate from the BL membrane either by disrupt-

ing tight junctions or by transcytosis. To test whether the integrity of the tight junction was affected, we assayed permeability to FITC-inulin, a low molecular weight molecule that is not able to diffuse through functional tight junctions (Apodaca et al., 1995). In control experiments, the monolayer was exposed to 10 mM EDTA for 10 min, a treatment known to disrupt AP junctions. Diffusion of FITC-inulin from the AP to the BL compartment was observed within 20 min and continued to increase for up to 60 min. In contrast, AP addition of bacteria did not increase the diffusion of FITC-inulin compared with uninfected cells. Instead, less FITC-inulin diffusion was observed, suggesting that bacteria may increase the stability of tight junctions (Fig. 4 b).

We confirmed these findings by testing whether in the absence of cell permeabilization the AP addition of antibodies to an extracellular domain of the BL protein β 1-integrin was able to access to the BL membrane. As seen in Fig. 4 c, AP addition of the β 1-integrin antibody only stained the AP surface at the site of the bacteria-associated protrusion. No BL staining was observed, confirming that *P. aeruginosa* did not disrupt the gate function of the adjacent tight junction. Together, these experiments strongly suggest that the *P. aeruginosa*-induced rerouting of BL markers does not occur by disruption of tight junctions.

We tested whether a dominant-negative (DN) mutant of dynamin II, which has previously been shown to inhibit BL endocytosis in MDCK cells (Altschuler et al., 1998), blocked the relocalization of BL proteins to the bacteria-induced AP protrusion.

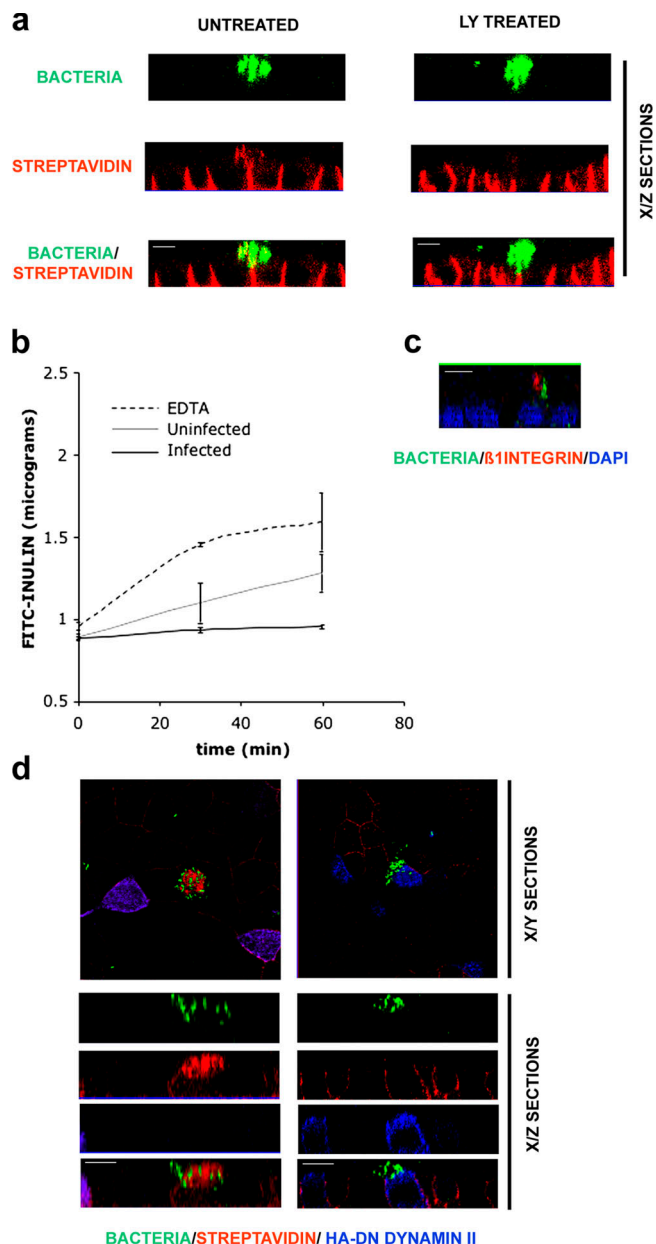


Figure 4. *P. aeruginosa* induces the relocalization of proteins from the BL to the AP surface by transcytosis. (a, left) The BL surface of confluent MDCK cells was biotinylated for 30 min at 4°C. Cells were infected with *P. aeruginosa* expressing GFP for 30 min at 37°C, fixed, and the AP and BL surfaces were probed with fluorescent streptavidin (red). Biotinylated BL proteins are only detectable on the BL surface and at the sites of bacterial attachment on the AP surface. (a, right) The relocalization of BL proteins is reduced by LY. Cells were preincubated with 100 μ M LY for 1 h. The drug was also present during biotinylation and infection. (b) The diffusion from the AP to the BL surface of the small molecule FITC-inulin was measured in uninfected cells, cells infected with *P. aeruginosa* for 30 min, or after exposure to 10 mM EDTA for 10 min. AP addition of *P. aeruginosa* decreased the monolayer permeability. Error bars represent SD. (c) XZ section of MDCK cells infected with GFP-*P. aeruginosa* and stained with an antibody against an extracellular domain of the BL protein β 1-integrin (red). The cells were then fixed and stained with DAPI (blue) to visualize the nuclei. The addition of bacteria did not permit diffusion of the antibody to the BL surface, suggesting that the tight junctions were not disrupted upon bacterial binding. (d) BL surface biotinylation of MDCK T23 cells previously infected with recombinant adenovirus encoding HA-tagged DN dynamin II was performed as described in panel a. DN dynamin II (blue) is expressed both in AP and BL membranes. The left panel shows a typical example of the relocalization of biotinylated BL proteins to the site of AP bacterial attachment

Infection of MDCK cells with an adenovirus construct expressing the DN dynamin II (K44A) under the control of a tetracycline-sensitive repressor prevented the accumulation of BL proteins at sites where *P. aeruginosa* bound to the AP surface (Fig. 4 d). Compared with nontransfected cells in the same sample ($n = 13$), relocalization of BL proteins to the site of AP binding was inhibited in cells expressing DN dynamin II by 56% ($n = 14$; $P < 0.01$ by chi-squared analysis).

LY has been shown to inhibit BL to AP transcytosis of ricin or dimeric IgA (Cardone and Mostov, 1995; Hansen et al., 1995). Consistent with our observed role for transcytosis, LY also inhibited the accumulation of biotinylated BL proteins at the site of AP bacterial binding (Fig. 4 a). Compared with cells treated with DMSO alone ($n = 12$), LY prevented the relocalization of biotinylated BL proteins by 54% ($n = 13$; $P < 0.01$ by chi-squared analysis).

Together, these results support the idea that a substantial amount of BL plasma membrane components are relocalized to the AP protrusion in a PI3K-dependent manner by transcytosis. We find no evidence that the bacteria disrupt the integrity of the tight junctions. We cannot exclude the possibility that some of the BL proteins found in protrusions are delivered from the Golgi in the biosynthetic pathway, although the long half-life of these proteins makes it unlikely that the amount of proteins available for biosynthetic delivery is large relative to the total intracellular pool.

We have recently shown that PIP3 is necessary and sufficient to specify BL membrane and that the exogenous addition of PIP3 transforms AP membrane in BL membrane by redirecting transcytosis (Gassama-Diagne et al., 2006). We have also reported that activation of the PI3K and Akt pathways are necessary and sufficient for *P. aeruginosa* internalization (Kierbel et al., 2005). The present study demonstrates that in the context of polarized MDCK cells, *P. aeruginosa*, an important human pathogen, subverts this pathway to create a local microenvironment that facilitates colonization and entry into the mucosal barrier. Similarly, binding of *P. aeruginosa* to the AP surface of the epithelial barrier is able to radically alter the composition of the membrane, transforming it from an AP surface to one with BL constituents. This transformation is accompanied by the recruitment of PI3K, the generation of PIP3, and the recruitment of actin into membrane protrusions. It is also possible that *P. aeruginosa* has additional ways to increase AP PIP3 such as by stimulating PIP3 diffusion and/or by decreasing the activity or levels of lipid phosphatases such as PTEN. In any case, by increasing AP PIP3, the bacteria appear to stimulate BL to AP transcytosis. For some molecules, Src kinase family-mediated phosphorylation inhibits BL recycling and allows cryptic AP sorting signals to predominate (Anderson et al., 2005). Interestingly, *P. aeruginosa* entry correlates with the activation of Src family kinases (Evans et al., 2002). We propose that as vesicles fuse with the AP membrane and release their cargo, AP constituents are pushed aside. In addition to promoting bacterial

to a nontransfected cell. The right panel shows that in cells expressing DN dynamin II, the AP accumulation of biotinylated proteins was inhibited. Bars, 10 μ m.

pathogenesis, the rerouting of BL molecules may also benefit the host by enhancing the innate immune response.

We found that the bacteria-induced protrusions were commonly found at cell–cell junctions. The close proximity to BL constituents at tight junctions may contribute to the rapid kinetics of the transformation of membrane polarity. This may also reflect the location of the host cell receptor. Although the cystic fibrosis membrane receptor and asialoganglioside GM1 have been suggested as receptors for entry (de Bentzmann et al., 1996; Pier et al., 1997), in the context of MDCK cells, at least, we consider them to be unlikely candidates and are actively investigating other candidate receptors.

We imagine that this subversion of host cell polarity is not absolute. Rather, in wounded or disrupted epithelium, the cells at the edge of the wound are poorly polarized, and *P. aeruginosa* is able to efficiently bind and coopt PI3K and generate PIP3 at the surface. As the mucosal barrier becomes increasingly differentiated and polarized, there is less binding (unpublished data), and the rerouting of BL markers to the AP surface is not as efficient, correlating with the decreased susceptibility of the intact epithelium to *P. aeruginosa*–mediated invasion or damage.

Materials and methods

Cell preparation and culture

MDCK cells (clone II, T23, or cells stably transfected with GFP-PH-Akt; Yu et al., 2005) were grown for 24 h on transwell filters as previously described (Kierbel et al., 2005). For transient transfections, MDCK cells were transfected with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The mammalian expression vector pEGFP containing the PH domain of Grp1 was a gift from M. Birnbaum (University of Pennsylvania, Philadelphia, PA). pEGFP-AH, which contains the PH domain of Akt, and the mutant AHR25C (both constructs encode amino acids 1–147 of Akt), in which the lipid-binding site is mutated (Watton and Downward, 1999), were gifts from J. Downward (London Research Institute, London, UK).

Antibodies and reagents

Antibodies were obtained from the following sources: PI3K antibodies were purchased from Transduction Laboratories; HA antibody was purchased from Santa Cruz Biotechnology, Inc.; β 1-integrin was obtained from the American Type Culture Collection; p58 antibody was a gift from K. Matlin (University of Cincinnati, Cincinnati, OH); anti-ZO-1 (R40.76) was a gift from B. Stevenson (University of Edmonton, Alberta, Canada); and gp135 antibody was a gift from G. Ojakian (State University of New York Downstate Medical Center, Brooklyn, NY). Secondary antibodies used were AlexaFluor594- or 647-conjugated antibodies obtained from Invitrogen. Actin filaments were stained with AlexaFluor350-phalloidin (Invitrogen). Streptavidin and AlexaFluor594 were obtained from Invitrogen. LY and latrunculin B were purchased from Calbiochem.

Immunofluorescence staining and image analysis

Immunofluorescent staining was performed as previously described (Apodaca et al., 1994). Samples were examined with a confocal microscope (LSM 510; Carl Zeiss Microimaging, Inc.) using a plan Apochromat 63 \times oil objective (Carl Zeiss Microimaging, Inc.). Images were acquired using Meta 510 software (Carl Zeiss Microimaging, Inc.), collected as TIFF files, and further analyzed with Photoshop 7.0 software (Adobe).

Bacterial infection

The *P. aeruginosa* strain PAK (obtained from J. Mattick, University of Queensland, Brisbane, Australia) was routinely grown shaking overnight in Luria-Bertani broth at 37°C. These stationary phase bacteria were labeled with the Red Fluorescent Cell Linker Mini kit (PKH26; Sigma-Aldrich), added at an MOI of 250 to MDCK cells plated at a density of 7×10^5 cells on 12-mm transwells (Costar), and grown for 24 h. After 30 min of infection at 37°C, cells were washed three times with PBS, fixed as previously described (Apodaca et al., 1994), and analyzed by confocal microscopy.

Similar results were seen at lower MOIs, but fewer bacterial aggregates bound to cells were observed.

Scanning microscopy

Filter-grown confluent polarized MDCK cells grown as described in Cell preparation and culture were infected with *P. aeruginosa* with an MOI of 50. After 30 min of infection at 37°C, cells were washed three times with PBS and fixed with 2% cacodylate glutaraldehyde for 30 min and 2% aqueous osmium tetroxide for 30 min as primary and secondary fixatives, respectively. Samples were then rinsed with distilled water, dehydrated to 100% ethanol, and incubated with hexamethyldisilazane (Sigma-Aldrich) until dry. Dried samples were mounted on aluminum holders, sputter coated with gold palladium (20-nM coating), and examined with a scanning electron microscope (model 5410; JEOL).

Transcytosis assay

MDCK cells were grown as described in Cell preparation and culture, and BL membrane proteins were specifically biotinylated with sulfo-N-hydroxy-sulfosuccinimide-biotin at 4°C for 30 min as described previously (Lipschutz et al., 2001). The biotin solution was removed, and cells were washed with PBS and incubated at 37°C for 5 min, at which time bacteria were added to the AP surface for 30 min. Transcytosis of BL proteins to the AP surface was detected by adding streptavidin and AlexaFluor594 conjugate to the AP and BL media for 30 min at 37°C, and cells were analyzed by confocal microscopy as described in Immunofluorescence staining and image analysis. In some experiments, cells were preincubated with LY (100 μ M in DMSO) for 1 h. LY was also present during biotinylation and infection.

Transfection with DN dynamin II

Construction of recombinant adenovirus and infection were performed as previously described (Altschuler et al., 1998). MDCK T23 cells (Barth et al., 1997) were grown for 3 d on 12-mm transwells. 18 h before the assay, cells were washed five times with PBS lacking CaCl_2 and infected for 2 h with 0.14 μ l of virus stock per transwell (~ 40 plaque-forming units/cell) in PBS lacking CaCl_2 . The recombinant adenovirus carries a HA-tagged version of a DN dynamin II mutant (K44A). The monolayers were washed three times with MEM media supplemented with 5% FBS, further incubated in the same medium containing a low concentration (1 ng/ml) of doxycycline for 16 h, and titrated to allow sufficient expression of the DN dynamin II protein without obviously disrupting the integrity of the monolayer.

Transepithelial permeability

MDCK cells were grown as described in Cell preparation and culture and infected with *P. aeruginosa* for 30 min or exposed to 10 mM EDTA for 10 min. After washing with PBS, 100 μ g/ml of prewarmed PBS + FITC-inulin (Sigma-Aldrich) was added to the AP chamber, and prewarmed PBS was added to the BL chamber. Cells were incubated at 37°C, and 100- μ l samples were collected from the BL chamber every 30 min. Fluorescence was quantified using a fluorescence plate reader (excitation/emission wavelength at 480/530 nm; 70% gain; Cytofluor).

Statistical analysis

Chi-squared analysis was performed. $P < 0.05$ was considered statistically significant.

Online supplemental material

Fig. S1 shows that *P. aeruginosa*–induced AP protrusions contain Grp1-PH-GFP, a probe for PIP3. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200605142/DC1>.

We thank Henry Bourne, Eric Brown, Orion Weiner, and members of our laboratories for advice and comments on the paper.

This work was supported by National Institutes of Health grants (HL55980 to K. Mostov and J. Engel, DK067153 to K. Mostov, and AI053194 and AI06592 to J. Engel).

Submitted: 23 May 2006

Accepted: 5 March 2007

References

- Altschuler, Y., S.M. Barbas, L.J. Terlecky, K. Tang, S. Hardy, K.E. Mostov, and S.L. Schmid. 1998. Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. *J. Cell Biol.* 143:1871–1881.

- Anderson, E., S. Maday, J. Sfakianos, M. Hull, B. Winckler, D. Sheff, H. Folsch, and I. Mellman. 2005. Transcytosis of NgCAM in epithelial cells reflects differential signal recognition on the endocytic and secretory pathways. *J. Cell Biol.* 170:595–605.
- Apodaca, G., L.A. Katz, and K.E. Mostov. 1994. Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. *J. Cell Biol.* 125:67–86.
- Apodaca, G., M. Bomsel, R. Lindstedt, J. Engel, D. Frank, K. Mostov, and J. Wiener-Kronish. 1995. Characterization of *Pseudomonas aeruginosa*-induced MDCK cell injury: glycosylation defective host cells are resistant to bacterial killing. *Infect. Immun.* 63:1541–1551.
- Barth, A.I., A.L. Pollack, Y. Altschuler, K.E. Mostov, and W.J. Nelson. 1997. NH2-terminal deletion of β -catenin results in stable colocalization of mutant β -catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J. Cell Biol.* 136:693–706.
- Cardone, M., and K. Mostov. 1995. Wortmannin inhibits transcytosis of dimeric IgA by the polymeric immunoglobulin receptor. *FEBS Lett.* 376:74–76.
- Cossart, P., and P.J. Sansonetti. 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science.* 304:242–248.
- de Bentzmann, S., C. Plotkowski, and E. Puchelle. 1996. Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. *Am. J. Respir. Crit. Care Med.* 154:S155–S162.
- Engel, J.N. 2003. Molecular pathogenesis of acute *Pseudomonas aeruginosa* infections. In *Severe Infections Caused by Pseudomonas aeruginosa*. A.R. Hauser and J. Rello, editors. Kluwer Academic Publishers, Boston. 201–230.
- Evans, D.J., D.W. Frank, V. Finck-Barbancon, C. Wu, and S.M. Fleiszig. 1998. *Pseudomonas aeruginosa* invasion and cytotoxicity are independent events, both of which involve protein tyrosine kinase activity. *Infect. Immun.* 66:1453–1459.
- Evans, D.J., T.C. Kuo, M. Kwong, R. Van, and S.M. Fleiszig. 2002. Mutation of csk, encoding the C-terminal Src kinase, reduces *Pseudomonas aeruginosa* internalization by mammalian cells and enhances bacterial cytotoxicity. *Microb. Pathog.* 33:135–143.
- Gassama-Diagne, A., W. Yu, M. ter Beest, F. Martin-Belmonte, A. Kierbel, J. Engel, and K. Mostov. 2006. Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. *Nat. Cell Biol.* 8:963–970.
- Geiser, T., B. Kazmierczak, L. Garrity-Ryan, M. Matthay, and J. Engel. 2001. *Pseudomonas aeruginosa* ExoT inhibits in vitro lung epithelial wound repair. *Cell. Microbiol.* 3:223–236.
- Gibson, M.C., and N. Perrimon. 2003. Apicobasal polarization: epithelial form and function. *Curr. Opin. Cell Biol.* 15:747–752.
- Hansen, S.H., A. Olsson, and J.E. Casanova. 1995. Wortmannin, an inhibitor of phosphoinositide 3-kinase, inhibits transcytosis in polarized epithelial cells. *J. Biol. Chem.* 270:28425–28432.
- Kazmierczak, B., K. Mostov, and J. Engel. 2001. Interaction of bacterial pathogens with polarized epithelium. *Annu. Rev. Microbiol.* 55:407–435.
- Kazmierczak, B.I., K. Mostov, and J.N. Engel. 2004. Epithelial cell polarity alters Rho-GTPase responses to *Pseudomonas aeruginosa*. *Mol. Biol. Cell.* 15:411–419.
- Kierbel, A., A. Gassama-Diagne, K. Mostov, and J.N. Engel. 2005. The phosphoinositide-3-kinase-protein kinase B/Akt pathway is critical for *Pseudomonas aeruginosa* strain PAK internalization. *Mol. Biol. Cell.* 16:2577–2585.
- Klarlund, J.K., A. Guilherme, J.J. Holik, J.V. Virbasius, A. Chawla, and M.P. Czech. 1997. Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science.* 275:1927–1930.
- Klarlund, J.K., L.E. Rameh, L.C. Cantley, J.M. Buxton, J.J. Holik, C. Sakelis, V. Patki, S. Corvera, and M.P. Czech. 1998. Regulation of GRP1-catalyzed ADP ribosylation factor guanine nucleotide exchange by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 273:1859–1862.
- Lipschutz, J.H., L.E. O'Brien, Y. Altschuler, D. Avrahami, Y. Nguyen, K. Tang, and K.E. Mostov. 2001. Analysis of membrane traffic in polarized epithelial cells. In *Current Protocols in Cell Biology*. John Wiley & Sons Inc., New York.
- Mandell, G.L., J.E. Bennett, and R. Dolin. 2000. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. Churchill Livingstone, Philadelphia.
- Pier, G.B., M. Grout, and T.S. Zaidi. 1997. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the lung. *Proc. Natl. Acad. Sci. USA.* 94:12088–12093.
- Pinal, N., D.C. Goberdhan, L. Collinson, Y. Fujita, I.M. Cox, C. Wilson, and F. Pichaud. 2006. Regulated and polarized PtdIns(3,4,5)P₃ accumulation is essential for apical membrane morphogenesis in photoreceptor epithelial cells. *Curr. Biol.* 16:140–149.
- Pizarro-Cerda, J., and P. Cossart. 2004. Subversion of phosphoinositide metabolism by intracellular bacterial pathogens. *Nat. Cell Biol.* 6:1026–1033.
- Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell.* 112:453–465.
- Sadoul, K., J. Lang, C. Montecucco, U. Weller, R. Regazzi, S. Catsicas, C.B. Wollheim, and P.A. Halban. 1995. SNAP-25 is expressed in islets of Langerhans and is involved in insulin release. *J. Cell Biol.* 128:1019–1028.
- Vanhaesebroeck, B., and D.R. Alessi. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* 346:561–576.
- von Stein, W., A. Ramrath, A. Grimm, M. Muller-Borg, and A. Wodarz. 2005. Direct association of Bazooka/PAR-3 with the lipid phosphatase PTEN reveals a link between the PAR/aPKC complex and phosphoinositide signaling. *Development.* 132:1675–1686.
- Watton, S.J., and J. Downward. 1999. Akt/PKB localisation and 3' phosphoinositide generation at sites of epithelial cell-matrix and cell-cell interaction. *Curr. Biol.* 9:433–436.
- Wymann, M.P., M. Zvelebil, and M. Laffargue. 2003. Phosphoinositide 3-kinase signalling—which way to target? *Trends Pharmacol. Sci.* 24:366–376.
- Yu, W., L.E. O'Brien, F. Wang, H. Bourne, K.E. Mostov, and M.M. Zegers. 2003. Hepatocyte growth factor switches orientation of polarity and mode of movement during morphogenesis of multicellular epithelial structures. *Mol. Biol. Cell.* 14:748–763.
- Yu, W., A. Datta, P. Leroy, L.E. O'Brien, G. Mak, T.S. Jou, K.S. Matlin, K.E. Mostov, and M.M. Zegers. 2005. Beta1-integrin orients epithelial polarity via Rac1 and laminin. *Mol. Biol. Cell.* 16:433–445.